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Suppression of programmed cell death regulates the cyclical degeneration of organs in a colonial urochordate

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Abstract

The survival of animal tissues and organs is controlled through both activation and suppression of programmed cell death. In the colonial urochordate *Botryllus schlosseri*, the entire parental generation of zooids in a colony synchronously dies every week as the asexually derived generation of buds reaches functional maturity. This process, called takeover, involves massive programmed cell death (PCD) of zooid organs via apoptosis followed by programmed removal of cell corpses by blood phagocytes within approximately 1 day. We have previously reported that developing buds in conjunction with circulating phagocytes are key effectors of zooid resorption and macromolecular recycling during takeover, and as such engineer the reconstitution of a functional asexual generation every week [Lauzon, R.J., Ishizuka, K.J., Weissman, I.L., 2002. Cyclical generation and degeneration of organs in a colonial urochordate involves crosstalk between old and new: a model for development and regeneration. Dev. Biol. 249, 333–348]. Here, we demonstrate that zooid lifespan during cyclic blastogenesis is regulated by two independent signals: a bud-independent signal that activates zooid PCD and a bud-dependent, survival signal that acts in short-range fashion via the colonial vasculature. As zooids represent a transient, mass-produced commodity during *Botryllus* asexual development, PCD regulation in this animal via both activation and suppression enables it to remove and recycle its constituent zooids earlier when intra-colony resources are low, while maintaining the functional filter-feeding state when resources are adequate. We propose that this crosstalk mechanism between bud and parent optimizes survival of a *B. schlosseri* colony with each round of cyclic blastogenesis.

Keywords: Ascidian; Apoptosis; Asexual budding; *Botryllus*; Cyclic blastogenesis; Urochordate; Programmed cell death

Introduction

Programmed cell death (PCD) and removal are essential physiological processes in the life of all multicellular animals. The majority of physiological cell deaths occur by apoptosis, defined morphologically as the manifestation of a phylogenetically conserved, intracellular death program (reviewed in Strasser et al., 2000; Danial and Korsmeyer, 2004). Apoptosis entails an orderly dismantling of the cell followed by engulfment and disposal of its corpse (programmed cell removal) by phagocytic cells (Henson et al., 2001). During the course of animal development, apoptotic PCD serves to eliminate cells or structures that are no longer needed or potentially harmful to the host and is essential to the emergence of form and function (Jacobson et al., 1997; Seipp et al., 2001; Baehrecke, 2002). In mammals, mutations or targeted disruptions in various components of the cell death machinery lead to severe abnormalities that are often lethal in embryonic or perinatal development (Baehrecke, 2002; Danial and Korsmeyer, 2004). In adult tissues, PCD also plays a critical role in tissue homeostasis, helping to control cell numbers in the host. Many of the factors that regulate the balance between life and death are extracellular signals that either activate or suppress PCD (Raff, 1992). For instance, in the vertebrate immune system, binding of the FAS-ligand to its receptor induces apoptosis in the cell expressing the FAS receptor (Strasser et al., 2000). Conversely, in the developing nervous system, cell survival depends on neurotrophic factors that are secreted by the target cells they innervate (Raff, 1992).

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However, in most situations, a combination of both PCD-activating and suppressing signals ultimately determines the fate of a cell (Jacobson et al., 1997). For example, in the early mouse embryo, formation of the proamniotic cavity requires the interplay between a short-range endodermal death signal which induces cavitation via apoptotic PCD and a basement membrane-derived, survival signal which suppresses death of the columnar epithelial cells that line the cavity (Coucouvanis and Martin, 1995, 1999).

We study PCD and regeneration in colonial ascidians, a group of ancestral marine invertebrate chordates in which the larva is endowed with a notochord, dorsal nerve tube, segmented musculature and preformed pharyngeal openings or gill slits (Burighel and Cloney, 1997; Ruppert et al., 2004). These animals possess a remarkable capacity for regeneration and exhibit a great diversity of mechanisms for propagation and survival (Nakauchi and Kawamura, 1986; Kawamura and Sugino, 1999). In the botryllid ascidians, massive programmed cell death (PCD) occurs in cyclical and coordinated fashion within tissues and organs of adult individuals (zooids) in conjunction with blastogenic growth via asexual budding in the colony. The subject of this particular study is the budding ascidian *Botryllus schlosseri*, a cosmopolitan inhabitant of shallow waters and harbors worldwide (Berrill, 1950; Burighel and Cloney, 1997). Upon hatching from the mother colony, the free-swimming chordate tadpole attaches to a subtidal surface and undergoes metamorphosis into a juvenile form called an oozooid (Milkman, 1967; Sabbadin, 1969). The sessile oozooid produces genetically identical buds via asexual reproduction, which ultimately become adult individuals (zooids). Asexual development in *Botryllus*, called cyclic blastogenesis, occurs as part of a highly coordinated process throughout the colony every week (Sabbadin, 1969; Lauzon et al., 1992). The bud arises as an outgrowth from the ventro-lateral wall of the parent zooid and segregates into a three-layered structure that contains parental atrial epithelium, epidermis and blood (Pizon, 1893; Berrill, 1941). Throughout its development, the bud retains a direct connection to its parent by means of a vascularized epidermal peduncle (Burighel and Brunetti, 1971; Mukai et al., 1978), and is highly dependent on this connection for development of polarity, bilateral asymmetry and growth (Berrill, 1941; Sabbadin, 1958; Izzard, 1973; Sabbadin et al., 1975). Several primary buds may be produced by a zooid, and as they develop, these buds produce buds of their own (secondary buds). With each new blastogenic generation, the number of zooids in a colony increases and, in time, zooids arrange themselves in star-shaped modules called systems (Milkman, 1967; Sabbadin, 1969). The entire colony is embedded in a transparent, gelatinous tunic, and each zooid is a transient, autonomous feeding individual that is interconnected to other individuals through a network of extracorporeal blood vessels (Burighel and Brunetti, 1971; Mukai et al., 1978; Weissman, 2000). The three overlapping asexual generations (adult zooids, primary and secondary buds) exhibit developmental synchrony and thus behave as a highly integrated, physiological unit (Watanabe, 1953; Sabbadin et al., 1991; Sabbadin, 1994).

At the conclusion of each round of cyclic blastogenesis, the entire generation of adult zooids dies simultaneously as a new asexual generation of primary buds reaches functional maturity. This systemic death phase, called takeover, begins with contraction of the zooid mantle followed by closure of oral and excurrent siphons (Burighel and Schiavinato, 1984; Lauzon et al., 1992). During the resorption phase which follows contraction, organismic apoptosis occurs throughout the zooid viscera (heart, neural complex and digestive system) and invading blood phagocytes rapidly engulf cell corpses (Burighel and Schiavinato, 1984; Lauzon et al., 1993). Zooid resorption is completed in approximately 1 day. Thus, unlike vertebrate species in which the body is long-lived, and cells and tissues are replaced when needed, a *B. schlosseri* colony generates new bodies every week while disposing of up to half of its mass via PCD and removal. While the underlying reason for this life history strategy is unclear, only botryllid ascidians exhibit organismic death and regeneration simultaneously. This trait suggests that a high degree of crosstalk operates between individuals in a colony. Indeed, we previously reported that, when buds were surgically removed in a colony (budectomy), zooid resorption was severely curtailed (Lauzon et al., 2002). Furthermore, we demonstrated that developing buds, colonial blood vessels and circulating phagocytes collaborate in a colony-wide recycling mechanism in which the raw materials derived from zooid cell corpses are utilized towards organismic regeneration (Lauzon et al., 2002). In this study, we investigated how the budding mechanism in *Botryllus* impacted on the dynamics of cyclic blastogenesis. We have utilized a similar microsurgical approach to specifically address what role, if any, developing buds play in the timing mechanism that determines zooid lifespan and onset of PCD in a colony. Our findings indicate that the zooid cell death program is regulated through both activation and suppression.

Experimental procedures

Culture and observation of animals

Wild colonies of the colonial ascidian *B. schlosseri* were scraped off from algal blades attached to floating docks at the Monterey Bay marina (Monterey, CA) during the 2000–03 summers, and from the Eel Pond in Woods Hole (MA) or the Sandwich marina (MA) during the 2004–05 summers. Monterey and Woods Hole *Botryllus* represent the same species as they were previously shown to interbreed and yield fertile F1 progeny (Boyd et al., 1990). Colonies were subsequently attached to 5×7.5 cm glass microscope slides (Fisher Scientific Research, Pittsburgh, PA) and placed within running sea water tables at the Marine Biological laboratory (MBL) or 17-liter tanks at the Hopkins Marine Station (Pacific Grove, CA). For experiments carried out at MBL, wild colonies were successfully grown at 21°C using the raw sea water system flowing into MBL. In contrast, for Monterey experiments, free-swimming tadpole larvae were collected from sexually mature colonies onto 5×7.5 cm slides vertically placed within glass racks. Juvenile oozooids and adult colonies were subsequently reared within 17-liter standing sea water tanks at 18°C and fed daily with 2.5 ml/tank of Liquifry (Interpet Ltd., Surrey, England), as previously described (Boyd et al., 1986). Clonal replicates were generated from multisystem colonies by severing blood vessels and tunic matrix between zooid systems with a razor blade and placing the fragmented pieces onto individual 5×7.5 cm glass slides in a moisture chamber for 20 min. All photographs were taken using a Nikon Coolpix 995 digital camera (Nikon USA, Melville, NY).

Microsurgical procedures (budectomies)

All microsurgical manipulations were performed on the dorsal surface of the colony, as previously described (Lauzon et al., 2002). Observations were made from both ventral and dorsal surfaces at 6-hour intervals (and occasionally every 3 h) in unmanipulated and sham-operated controls and budectomized clonal replicates. Budectomies were carried out with the use of a Wheeler dissecting knife (Ernest Fullam Inc., Latham, NY) under a binocular Wild-Heerbrugg M5A (Technical Instruments Co., San Francisco, CA) or Zeiss SV-8 stereomicroscope (Carl Zeiss Inc., Thornwood, NY). Three types of budectomies were performed as follows: (a) complete budectomy: the tunic matrix and colonial blood vessels connecting zooid systems in multisystem colonies were severed between zooid systems at the onset of a new blastogenic cycle (stage A-1). All buds (primary and secondary) were removed from the small colony fragments (between 1 and 3 zooid systems) resulting from the cut, at various stages of cyclic blastogenesis ranging from A-2 through early C-2. Attempts were also made to budectomize zooid systems at stage A-1. However, new and/or dormant palleal buds were often observed to regenerate from the lateral wall of the budectomized parent zooids over the 24–48 h following budectomy. As a result of these inherent difficulties, stage A-1 surgeries were excluded from the study. Two types of clonal replicate controls were also utilized: unmanipulated and sham-operated controls. For unmanipulated controls, the only alteration brought about to the colony was the razor blade cut to isolate clonal replicates in stage A-1. This treatment had no effect on the length of the blastogenic cycle, when compared with other *B. schlosseri* colonies cultured on separate glass slides. For sham surgeries, the following surgical alterations were carried out: the tunic matrix isolating the zooids from the external sea water was severed, as were the radial blood vessel connecting primary bud to marginal vessel and the vascularized epidermal peduncle connecting primary bud and zooid. A blood clot was produced within seconds of the rupture of these vascular connections, and new tunic was synthesized within hours to repair the damaged colonial matrix. (b) Hemi-budectomy of multisystem colonies: for the majority of these surgeries, all buds were removed from half of the zooid systems present in a colony (typically between two and three systems), between stage A-2 and early C-2 of cyclic blastogenesis. Thus, in a colony made up of two systems of zooids, buds were removed from only one system. In colonies made up of three systems, buds were removed from two of the three systems. (c) Hemi-budectomy of single-system colonies: for these surgeries, all buds were removed between stage A-2 and early C-2, leaving only one dextral primary bud (and its attached secondary bud) connected to one of the zooids in a single system of zooids.

Histology

Zooid systems were fixed in 1% paraformaldehyde/filtered sea water (PFA/FSW) for 2 h at 4°C on an orbital shaker, as previously described (Lauzon et al., 1992, 2002). Briefly, they were subsequently washed twice sequentially in FSW and phosphate buffered saline (pH 7.4). Specimens were infiltrated overnight at 4°C and embedded in JB-4 Plus plastic (Polysciences Inc., Warrington, PA), according to the manufacturer's specifications. Serial sections 2 μm thick were cut with a Histoknife (Diatome Inc., Switzerland) along the zooid's antero-posterior axis, as previously described (Lauzon et al., 1992). Sections were adhered onto glass microscope slides and stained with a 0.05% toluidine blue/2.5% sodium bicarbonate solution, mounted with Permount (Fisher) and observed using a BH-2 model compound microscope (Olympus Corp., New York/New Jersey Inc., Middlebush, NJ).

Statistics

Owing to slight temporal differences in bud development and blastogenic cycle duration between Monterey and Woods Hole colonies, both populations were analyzed separately. Furthermore, median times of onset of PCD within experimental groups (complete budectomy, multisystem hemi-budectomy and single-system hemi-budectomy with one bud) were not normally distributed. Therefore, non-parametric Kruskal–Wallis (with Dunn's multiple comparisons for Monterey colonies) and Mann–Whitney tests (for Woods Hole colonies) were used in lieu of one-way analysis of variance (ANOVA). A critical value of $p<0.05$ was used to evaluate significant differences between groups. All statistical analyses were carried out using the GraphPad InStat software package (GraphPad software, Inc., San Diego, CA).

Results

Overview of cyclic blastogenesis in B. schlosseri

The blastogenic cycle in *B. schlosseri* is divided into four stages, A through D, and each stage is further subdivided based on morphological characteristics of adult zooids, primary and secondary buds (Fig. 1; also see Berrill, 1941; Watanabe, 1953; Sabbadin, 1958; Izzard, 1973; Lauzon et al., 2002). The length of each cycle is temperature-dependent, lasting approximately 7 days at 18°C (Monterey colonies) and 5 days at 21°C (Woods Hole colonies). During stage A-1, secondary bud formation is initiated with the onset of a new blastogenic cycle. It evaginates from the primary bud's lateral wall to form a prominent hemisphere. By stage A-2, the hemisphere exhibits a skewing towards the anterior end of the parent zooid, and when the colony reaches stage B-1, heartbeat begins within the primary bud. During stage B-2, a primary blood circulation is established within the secondary bud as it forms a closed, double-layered vesicle. Organogenesis is orchestrated during stage C-1 by the formation of a gut rudiment at the future posterior end, just prior to the elaboration of two atrial folds at the future anterior end. The primary subdivisions, which delineate a central, branchial chamber from two lateral, peribranchial chambers are completed by early stage C-2. By mid-to-late C-2, a pericardial rudiment is formed on the right posterior side and pigmentation of the primary bud intensifies (Fig. 2A). In stage D or takeover, all adult zooids synchronously begin to die. In early takeover (stage D-1), the zooids undergo periodic contractions for approximately 3 h, close both oral and excurrent siphons, lose responsiveness to mechanical stimuli as well as body wall pigmentation (Fig. 2B). Following contraction (stage D-2), the characteristic star-shaped arrangement of systems is lost as zooids separate from each other in the colony (Fig. 2C). By mid-takeover (stage D-3), zooids are actively being resorbed (Fig. 2D). During this period, the primary buds undergo a dramatic growth surge and gradually assemble into the characteristic star-shaped systems as they become functionally mature (Figs. 2B–D). A new blastogenic cycle begins with the opening of siphons and filter-feeding in the new adult zooid generation (not shown). Development from birth of a secondary bud to death of a zooid requires the completion of three blastogenic generations.

Complete budectomy induces early onset zooid PCD

In a previous study, we reported that budectomy severely impaired zooid resorption during takeover (Lauzon et al., 2002). During the course of those experiments, we had also observed that many of the budectomized colonies appeared to undergo regressive changes earlier than staged-matched controls (RJL, unpublished observations). This unexpected finding implied that developing buds could affect zooid survival during cyclic

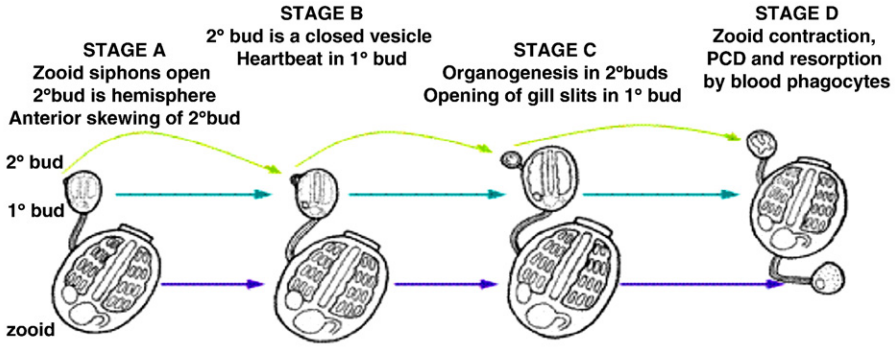

Fig. 1. Diagram of cyclic blastogenesis in *B. schlosseri* delineating stages A through D (adapted from Watanabe, 1953). Each colony is made up of three overlapping asexual generations (adult zooids, primary and secondary buds). Each asexual generation of individuals exhibits developmental synchrony. Abbreviations: 1° bud, primary bud; 2° bud, secondary bud.

blastogenesis. However, we could not rule out the possibility that these changes resulted from developmental desynchronization that can occur once the vascular connections are ruptured between clonal replicates of multisystem colonies. Thus, in order to properly address whether budectomy affected onset of PCD and zooid lifespan during cyclic blastogenesis, a prospective series of experiments with stage-matched, unmanipulated and sham-operated controls was carried out. In order to minimize desynchronization between clonal replicates, vascular connections between zooid systems of multisystem colonies were severed in stage A-1 as the new functional zooids opened their siphons. Sham surgeries were also carried out to rule out

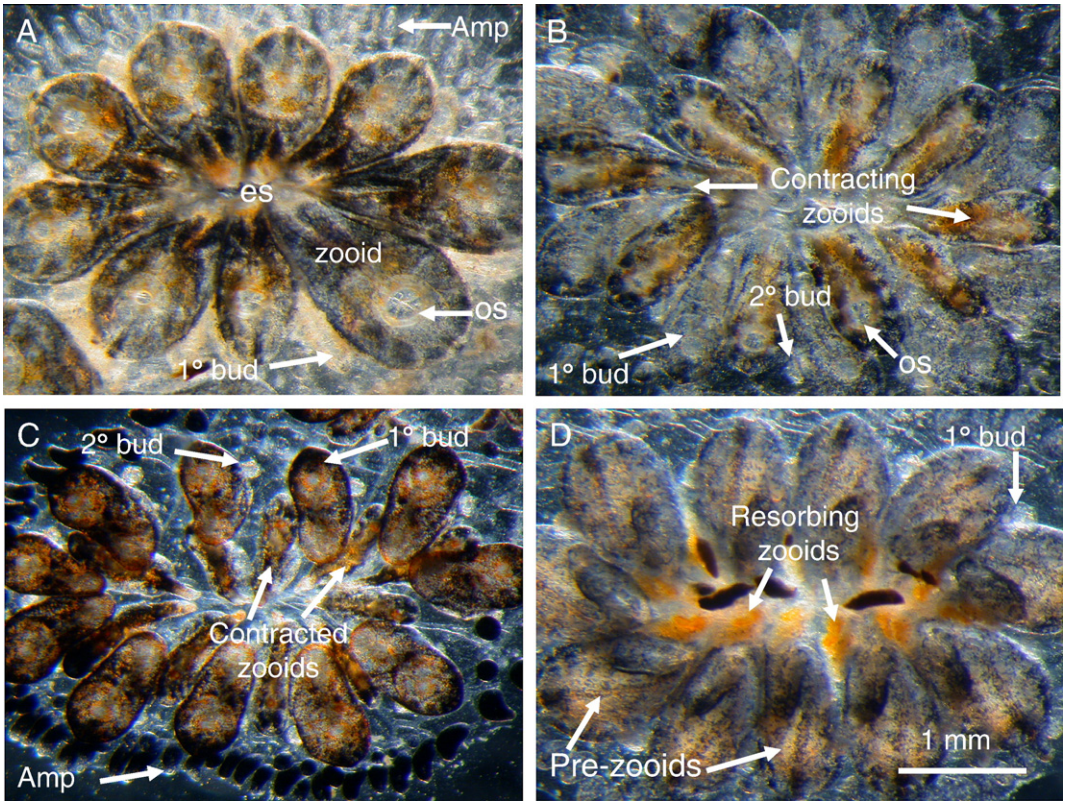

Fig. 2. The takeover phase of cyclic blastogenesis in *B. schlosseri*. (A) Mid-stage C-2 of cyclic blastogenesis, approximately 24 h prior to the onset of takeover, depicting filter-feeding zooids with open oral and excurrent siphons. The primary buds are beginning to accumulate pigment cells in their mantle. The ampullae delineate the colony's periphery. (B) Stage D-1: the generation of adult zooids is actively contracting. At this stage, which lasts approximately 3 h, the oral siphons of most zooids are still open and responsive to mechanical stimulus. The primary buds have accumulated more pigment cells and are approximately the size of the regressing adult zooids. (C) Stage D-2: the regressing adult zooids have completed their contraction phase, are no longer responsive to mechanical stimulus and siphons are closed. As the zooids begin their dissolution, the primary buds grow larger in size. The peripheral ampullae serve as transient storage sites for pigment cells until primary buds reach functional maturity. (D) Stage D-3: zooid resorption. Remnants of the digestive system and endostyle are still visible, and the heart is still beating. The primary buds are now prefunctional zooids, whereas the secondary buds have become primary buds. Panels A–C represent dorsal views, whereas panel D is a ventral view. Abbreviations: 1° bud, primary bud; 2° bud, secondary bud; Amp, ampullae; es, excurrent siphon; os, oral siphon. Scale bar indicates 1 mm.

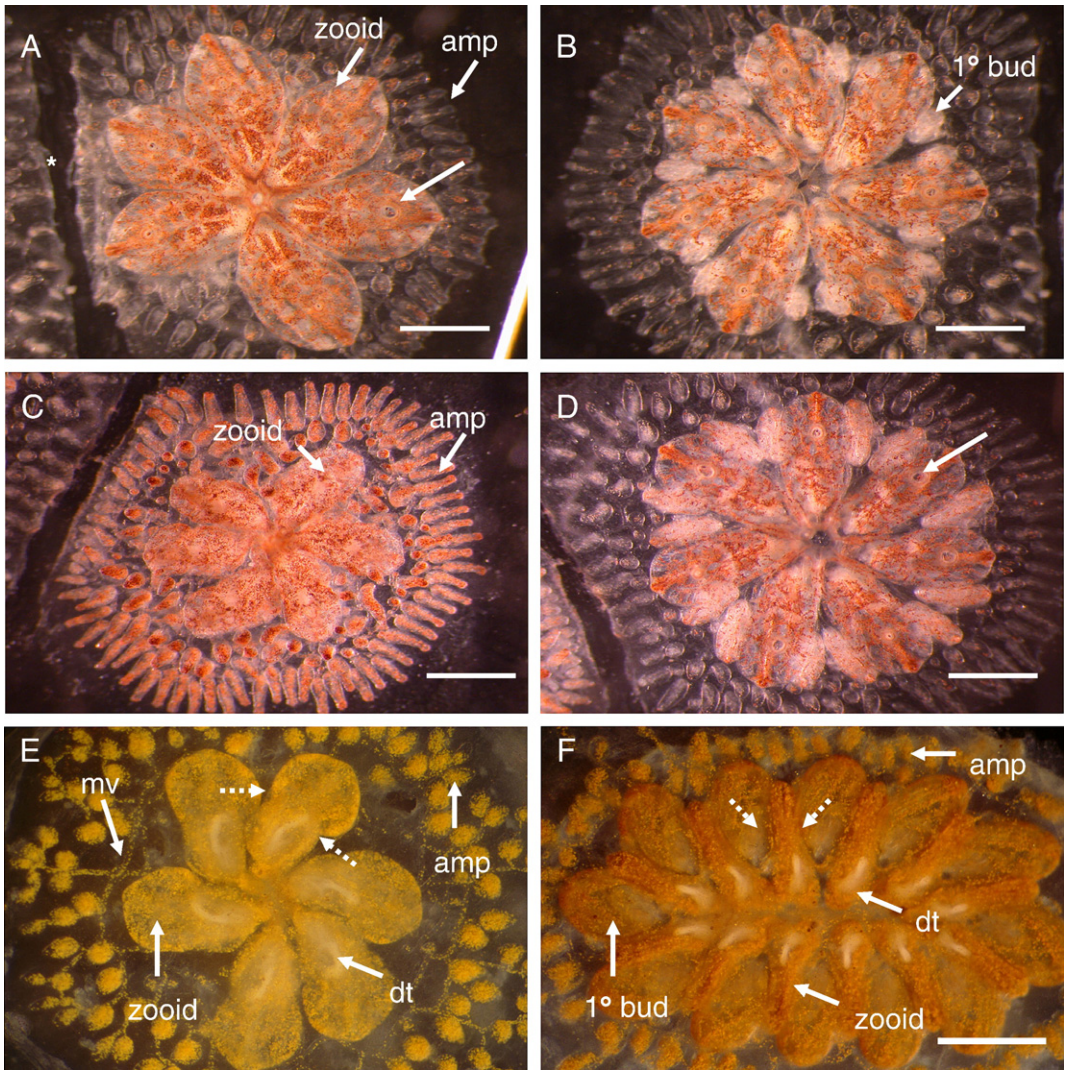

Fig. 3. Complete budectomy induces early onset of PCD in Monterey *B. schlosseri*. Vascular connections were severed between systems in a colony made up of two systems of zooids (asterisk denotes position of the cut) at the onset of a new blastogenic cycle (stage A-1). Complete budectomy was carried out on one system of zooids at the B-1 stage of cyclic blastogenesis. (A) System of zooids 24 h following complete budectomy. The zooids are inflated and actively filter-feeding. The arrow denotes an open oral siphon from one of the zooids in this colony. (B) Control clonal replicate photographed at the same time as budectomized clone, at stage B-2 of cyclic blastogenesis. (C) Same budectomized system of zooids as in panel A, exhibiting early onset of PCD, approximately 24 h prior to its control clonal replicate. Please note that the budectomized zooids have contracted, shutdown their siphons and undergone a partial loss of pigmentation in their mantle wall. Pigment cells have begun to accumulate in the peripheral ampullae. (D) Same control clonal replicate as in panel B, photographed at the same time as the budectomized clone, in early stage C-2 of cyclic blastogenesis approximately 24 h before its onset of takeover. Arrow depicts an open oral siphon of a filter-feeding zooid. (E) Budectomized colony made up of one system of zooids at the onset of takeover, viewed from the ventral plane. (F) Stage-matched, control colony made up of one system of zooids at the onset of takeover, viewed from the ventral plane. Note the bilateral contraction of the zooid mantle wall (dashed arrows) in both control and budectomized zooids. The digestive tract (dt) outlines the posterior end of each zooid. Panels A–D represent dorsal views, whereas panels E and F are ventral. Abbreviations: 1° bud, primary bud; amp, ampulla; dt, digestive tract; mv, marginal vessel. Scale bar indicates 1 mm.

the possibility that mechanical stress resulting from surgery could shorten the blastogenic cycle non-specifically. All of the colonies manipulated in this fashion remained stage-synchronized during the blastogenic cycle in which observations were performed. Furthermore, budectomy did not exert any adverse effect on the colony as zooids remained functional, filter-feeding units often several days following surgery (Figs. 3A, B). Blastogenic cycle duration varied with growth temperature (5 and 7 days for Woods Hole and Monterey colonies, respectively), but no additional differences in asexual development were noted between the two *B. schlosseri* populations. A combined total of 45 complete budectomies were carried out on both Monterey and Woods Hole colonies and compared to stage-synchronized clonal replicates. Thirty five colonies subjected to complete budectomy between stage A-2 and B-2 exhibited an early onset of zooid PCD, of which 30 (85.7%) underwent PCD in early stage C-2, approximately 24–36 h prior to onset of takeover in unmanipulated and sham-operated control clones (Tables 1 and 2, Fig. 4). Morphologically, budectomized zooids undergoing PCD initiated a series of periodic contractions and partial loss of pigmentation of the mantle wall and eventually closed both oral and excurrent

Table 1
Summary of budectomies in Monterey *Botryllus schlosseri* colonies

Type of surgery	Number of zooid systems	Number of surgeries	Stage of surgery				
			A2	B1	B2	C1	Early C2
Complete budectomy	1	20	7	8	3	2	0
[*N*=24]	Early onset of PCD[a] (24–36 h)	16	6	7	3	0	0
	Early onset of PCD (12–24 h)	2	1	1	0	0	0
	2	4	0	1	2	1	0
	Early onset of PCD (24–36 h)	3	0	1	2	0	0
	Early onset of PCD (12–24 h)	0	0	0	0	0	0
Hemi-budectomy multisystem	2	47	11	12	7	13	4
	Bud/zooid ratio±SD[b]		0.53±0.19	0.60±0.11	0.62±0.24	0.54±0.14	0.54±0.06
[*N*=66]	Early onset of PCD (24–36 h)	0	0	0	0	0	0
	Early onset of PCD (12–24 h)	6	1	4	1	0	0
	Early onset of PCD (6–12 h)	4	1	1	2	0	0
	3	19	3	5	8	1	2
	Bud/zooid ratio±SD		0.45±0.16	0.66±0.14	0.52±0.16	0.59±0	0.46±0.11
	Early onset of PCD (24–36 h)	0	0	0	0	0	0
	Early onset of PCD (12–24 h)	5	0	3	2	0	0
	Early onset of PCD (6–12 h)	2	1	0	1	0	0
Hemi-budectomy	1	41	9	10	10	6	6
(one bud remains)	Bud/zooid ratio±SD		0.13±0.05	0.14±0.06	0.19±0.06	0.12±0.04	0.16±0.09
[*N*=41]	Early onset of PCD (24–36 h)	0	0	0	0	0	0
	Early onset of PCD (12–24 h)	12	5	3	4	0	0
	Early onset of PCD (6–12 h)	8	2	3	3	0	0
	PCD gradient[c]	19	7	6	6	0	0

[a] As determined via stereomicroscopy by contraction and loss of pigment in zooid mantle wall, shutdown of oral and excurrent siphons and loss of responsiveness to mechanical stimulus.
[b] Following budectomy.
[c] Budectomized zooids within system undergo PCD, while zooid with connected primary bud retains functional filter-feeding state as long as unmanipulated controls.

siphons with a concomitant loss of responsiveness to mechanical stimuli (Figs. 3C, D). When viewed from their ventral side, budectomized zooids displayed a prominent bilateral contraction along the mid-plane halfway between the anterior and posterior end (Fig. 3E). In contrast, zooids from control colonies exhibited a prominent anterior constriction and appeared as if 'squeezed' by the dorsal migration of buds during their period of rapid growth during takeover (Fig. 3F). In addition, budectomized zooids failed to resorb properly when compared to controls (Fig. 4). Histological observations further revealed that tissues and organs of budectomized zooids appeared morphologically intact prior to the initiation of PCD (Figs. 5A, B). However, by 36 h post-onset of PCD, despite lack of significant macroscopic zooid resorption, numerous blood phagocytes with engulfed cell corpses were observed in the peribranchial cavity of budectomized zooids (Fig. 5C). Zooids from control colonies also displayed similar regressive changes, albeit by 12 h post-onset of takeover (Fig. 5D). The remaining 10 colonies budectomized in stage C-1 initiated zooid PCD in concert with that of controls (Tables 1 and 2).

Parabiotic vascular connections rescue premature onset of zooid PCD in hemi-budectomized colonies

The above-described findings indicate that, although zooids are competent to die in the complete absence of buds, the timing mechanism that determines proper onset of PCD operates in a developmental stage-dependent fashion which requires the presence of buds. We next sought to investigate the functional involvement of the colonial vasculature in this timing mechanism. Buds and zooids are interconnected by means of extracorporeal blood vessels that integrate the colony physiologically (Watanabe, 1953; Sabbadin et al., 1991; Lauzon et al., 2002). It is possible that a survival signal could originate from developing buds and suppress early onset zooid PCD via the vasculature. In order to

Table 2
Summary of budectomies in Woods Hole *Botryllus schlosseri* colonies

Type of surgery	Number of zooid systems	Number of surgeries	Stage of surgery		
			B1	B2	C1
Complete budectomy [*N*=21]	1	10	5	2	3
	Early onset of PCD[a] (≈24 h)	6	5	1	0
	Early onset of PCD (≈12 h)	1	0	1	0
	Early onset of PCD (≈6 h)	0	0	0	0
	2	9	0	5	4
	Early onset of PCD (≈24 h)	4	0	4	0
	Early onset of PCD (≈12 h)	1	0	1	0
	Early onset of PCD (≈6 h)	0	0	0	0
	3	2	1	1	0
	Early onset of PCD (≈24 h)	1	1	0	0
	Early onset of PCD (≈12 h)	0	0	0	0
	Early onset of PCD (≈6 h)	1	0	1	0
Hemi-budectomy multisystem [*N*=24]	2	21	10	10	1
	Bud/zooid ratio±SD[b]		0.62±0.12	0.62±0.12	0.57±0
	Early onset of PCD (12 or 24 h)	0	0	0	0
	Early onset of PCD (≈6 h)	3	2	1	0
	3	3	2	1	0
	Bud/zooid ratio±SD		0.43±0.25	0.41±0	0
	Early onset of PCD (12 or 24 h)	0	0	0	0
	Early onset of PCD (≈6 h)	1	0	1	0

[a] As determined via stereomicroscopy by contraction and loss of pigment in zooid mantle wall, shutdown of oral and excurrent siphons and loss of responsiveness to mechanical stimulus.
[b] Following budectomy.

address this question specifically, hemi-budectomies were carried out on colonies made up of 2–3 systems each. A total combined 90 multisystem hemi-budectomies were performed on both Monterey and Woods Hole colonies. All 20 colonies hemi-budectomized between stage C-1 and early C-2 exhibited synchronous onset of PCD within the budectomized and control zooid systems (Tables 1 and 2). Of the remaining 70 colonies that were hemi-budectomized between stage A-2 and B-2, 49 colonies (70%) initiated zooid PCD simultaneously with unmanipulated and sham-operated controls (Tables 1 and 2). Budectomized zooid systems from hemi-budectomized colonies exhibited regressive changes (mantle contraction, siphon shutdown) that were indistinguishable morphologically from the control zooid systems (Figs. 6A, B). In addition, budectomized and control zooid systems were resorbed in concert with each other within approximately 48 h (Figs. 6C, D). Of the remaining 21 colonies hemi-budectomized between stage A-2 and B-2 (30%), 11 colonies (15.7%) exhibited early onset zooid PCD during the mid-C-2 stage, approximately 12–24 h prior to onset of takeover in control clonal replicates (Table 1). The remaining 10 colonies (14.3%) underwent early PCD in late C-2 stage, approximately 6–12 h prior to controls (Tables 1 and 2).

Rescue of early onset zooid PCD in hemi-budectomized colonies is dependent on bud proximity

Our findings thus far indicate that a bud-dependent survival signal transmitted through the vasculature is functionally involved in suppressing early zooid PCD during takeover. Furthermore, the data strongly suggest that the critical period occurs between stage A-2 and B-2 as stage C colonies become refractory to budectomy-induced early onset of PCD. We thus proceeded to analyze hemi-budectomies carried out between stage A-2 and B-2 with greater scrutiny. Of the 50 combined Monterey and Woods Hole, two-system hemi-budectomized colonies, 13 (26%) exhibited an early onset of zooid PCD. By comparison, 8 of 19 three-system colonies (42%) underwent premature zooid PCD (Tables 1 and 2). Interestingly, in the great majority of the three-system hemi-budectomies, PCD occurred in a gradient fashion: the zooid system that was furthest removed from the control system of zooids underwent early zooid PCD first (Figs. 7A, B). In the example depicted, the zooid system on the right (#1) initiated mantle wall contractions and siphon shutdown approximately 10 h before the central (#2) zooid system (Fig. 7B). In turn, the central zooid system initiated takeover approximately 11 h before the control zooid system (#3) on the right side of the colony (Fig. 7C).

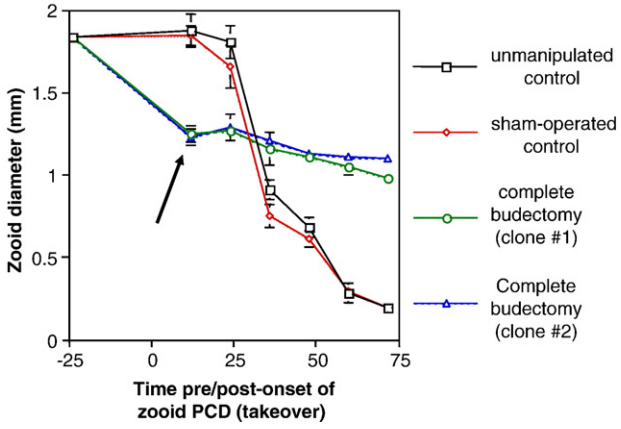

Fig. 4. Complete budectomy induces early onset zooid programmed cell death (PCD) and curtails zooid resorption. Complete budectomy was carried out at the B-1 stage of cyclic blastogenesis using two clonal replicates of the 1025F-3 Monterey *Botryllus* colony. When compared to unmanipulated and sham-operated control clonal replicates, onset of PCD in the budectomized clones (as assessed morphologically by siphon closure, zooid contraction and loss of responsiveness to mechanical stimuli) occurred prematurely by approximately 24 h (arrow), at the C-2 stage of cyclic blastogenesis. Zooid resorption in budectomized colonies was also severely curtailed.

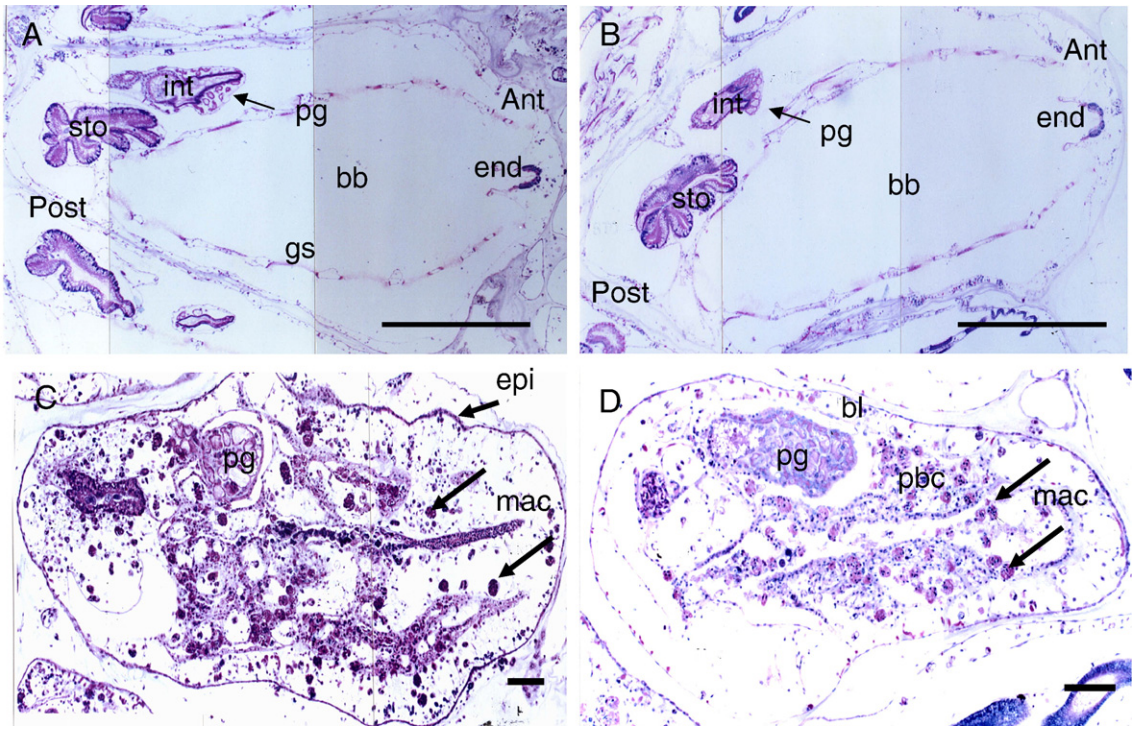

Fig. 5. Histological analysis of budectomized Woods Hole *B. schlosseri* colonies. (A) Thin section in the dorsal plane of a zooid 24 h following complete budectomy, at stage B-2 of cyclic blastogenesis. Note the retention of normal tissue and organ morphology in budectomized zooid. (B) Thin section in the dorsal plane of a zooid in a control unmanipulated clonal replicate, at stage B-2 of cyclic blastogenesis. (C) Thin section in the dorsal plane of a zooid from a budectomized colony, approximately 36 h post-onset of PCD. (D) Thin section in the mid-dorsal plane of a zooid from a control clone, approximately 15 h post-onset of takeover. Note the presence of numerous macrophages which have invaded the degenerating peribranchial cavity, in both budectomized and control zooids. Abbreviations: Ant, anterior end of zooid; bb, branchial basket; bl, blood sinus; end, endostyle; epi, epidermis; gs, gill slit; int, intestine; pbc, peribranchial cavity; mac, macrophages; pg, pyloric gland; Post, posterior end of zooid; sto, stomach. Scale bar indicates 0.5 mm (A, B) and 50 μm (C, D).

Rescue of zooid PCD in hemi-budectomized colonies is dependent on bud dosage

All of the two- and three-system hemi-budectomies described above displayed comparable bud/zooid ratios following budectomy (Tables 1 and 2) and offered little insight as to the minimal number of buds required to exert a suppressive effect on zooid PCD. Thus, in order to determine if bud dosage was critical to PCD suppression, single-system hemi-budectomies were carried out at various stages of cyclic blastogenesis, leaving only a single, developing primary (and secondary) bud. From a total of 41 Monterey *Botryllus* colonies, 20 of 29 colonies (69%) budectomized between stage A-2 and B-2 initiated early onset PCD in mid–late C-2. None died in early C-2. Furthermore, in 19 of these 20 colonies (95%), PCD occurred in a gradient fashion (Table 1). The zooid directly joined to a primary bud through a vascularized epidermal peduncle remained functional the longest, whereas other zooids contracted and closed their oral siphons (Fig. 7D). Using non-parametric tests, the difference in median time of onset of PCD in Monterey colonies subjected to complete budectomy was significantly lower than either multisystem, hemi-budectomized colonies or single-system hemi-budectomized colonies with one bud ($p<0.0001$). On the other hand, the difference in median time of onset of PCD between the latter two groups was not statistically significant ($p>0.05$). For Woods Hole colonies, the difference between the median time of onset of PCD in colonies subjected to complete budectomy was significantly lower than multisystem, hemi-budectomized colonies ($p<0.0001$).

Early onset of zooid PCD is regulated by size of developing buds

The observed stage specificity of PCD suppression suggests that the size of bud, possibly by virtue of its differentiation state, may be instrumental at regulating this timing mechanism. Over the years, during the course of routine observations on laboratory mariculture-raised Monterey *Botryllus*, we have noted that older colonies can display shortened blastogenic cycles (RJL, unpublished observations). Sabbadin (1956, 1958, 1994) has also documented a similar phenomenon in his laboratory stocks. He observed that severe environmental conditions could induce the adult generation to undergo premature regression, thus transiently reducing the number of coexisting generations to two. A two-year-old colony exhibiting premature takeover (clonal replicates of colony 1225c) was followed for three blastogenic cycles (Figs. 8 and 9). When compared to a morphologically normal control clone (1225c-29), two clonal replicates (1225c-11a and -11b) initiated premature takeover at the C1 stage of the

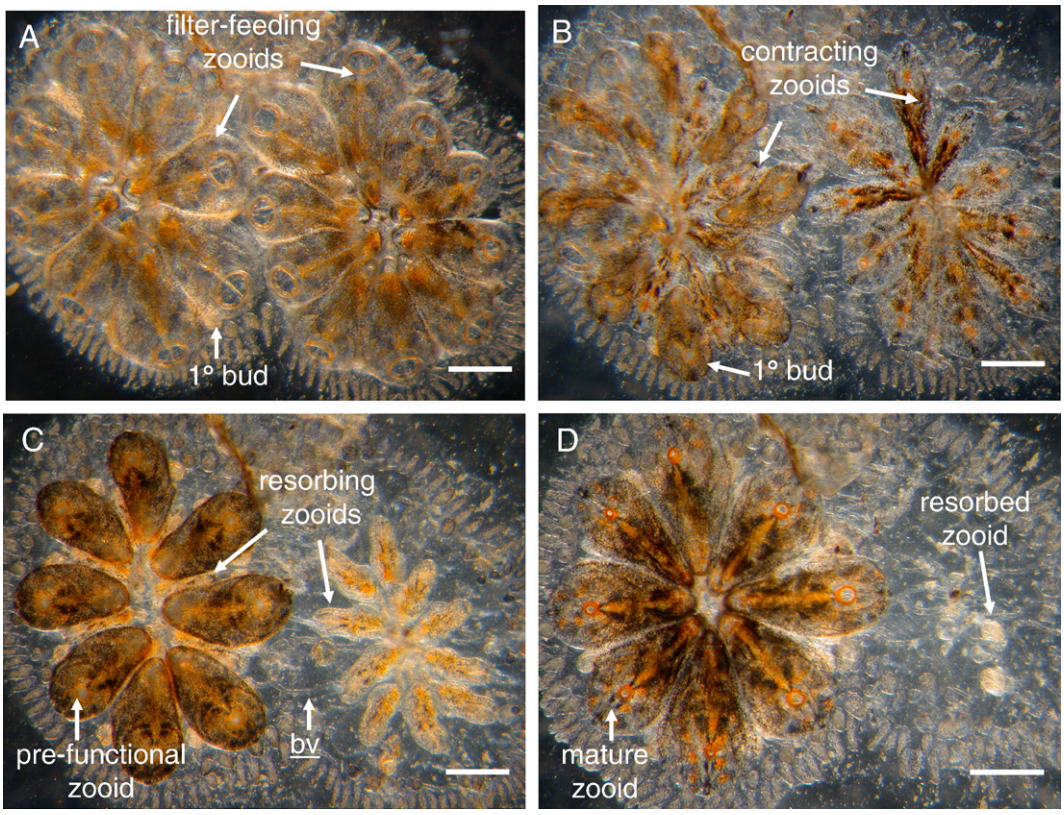

Fig. 6. Synchronous onset of PCD in hemi-budectomized *B. schlosseri* colonies. A representative Woods Hole (MA) colony made up of two systems of zooids, in which buds were removed only from the system on the right side between stage B-1 and B-2 of cyclic blastogenesis. Both systems are connected by extracorporeal blood vessels. (A) Approximately 20 h following hemi-budectomy, the colony is in stage C-1 and all zooids are actively filter-feeding. (B) Onset of takeover occurs simultaneously in both systems, approximately 25 h later. Note that zooids from both systems are actively contracting. (C) 45 h later, zooids from both systems are getting resorbed simultaneously. (D) 70 h later, the 'old' zooid generation is completely resorbed and mature zooids of the new asexual generation are filter-feeding. Note that the size of individual zooids is larger in the latter generation of zooids and that the pigmentation in their mantle wall is more pronounced. Panels A–D represent dorsal views. Abbreviations: 1° bud; primary bud; bv, blood vessel. Scale bar indicates 1 mm.

first blastogenic cycle (cycle *N*). Interestingly, all of the zooids in both colonies exhibited undersized primary buds (Figs. 8A and 9). Despite the fact that zooids were normally resorbed in approximately 24–36 h (Fig. 8B), the duration of this first cycle was 5 days, as opposed to 7 in the control clone 1225c-29. During the second blastogenic cycle which lasted 5.5 days (cycle *N*+1), takeover also occurred in asynchronous fashion at stage C1 (Fig. 8C). Despite that primary bud diameter was comparable to the control clone during this second cycle, several of the secondary buds failed to undergo the initial phases of organogenesis (Figs. 8D and 9). By the third blastogenic cycle (cycle *N*+2), bud diameter, organogenesis and onset of takeover all occurred normally (Figs. 8E, F, 9). Both clonal replicates resumed their normal, 7-day cycle.

Discussion

The current study was designed to investigate the functional involvement of buds in the regulation of zooid lifespan during cyclic blastogenesis in *B. schlosseri*. The simultaneous occurrence of massive organismic death and regeneration during the weekly change of asexual generations (i.e. takeover) suggests that both processes are tightly linked and could thus play an important role in colony homeostasis. We have demonstrated that two independent signals regulate proper onset of zooid PCD during takeover in a developmental stage-specific fashion: a bud-dependent survival signal suppresses PCD in the early stages of secondary bud organogenesis and a bud-independent signal activates PCD in adult zooids. PCD suppression occurs in short-range fashion involving the colonial vasculature and is dependent on the number of buds present in a colony (i.e. dosage). Our findings are consistent with a model in which survival signal expression may depend on nutritional and/or physiological cues regarding the overall health of the colony.

Zooid lifespan is regulated by PCD activation and suppression

Following complete budectomy, onset of zooid PCD always occurred prematurely, typically in early stage C-2 of cyclic blastogenesis, approximately 24–36 h prior to onset of takeover in stage-matched clonal replicates. Furthermore, early onset PCD was triggered in a developmental stage-specific manner as zooid lifespan was only curtailed when buds were removed up until stage B-2 of cyclic blastogenesis. Premature zooid PCD was never observed when budectomies were carried out in early stage C-1 or beyond. These observations are consistent with the

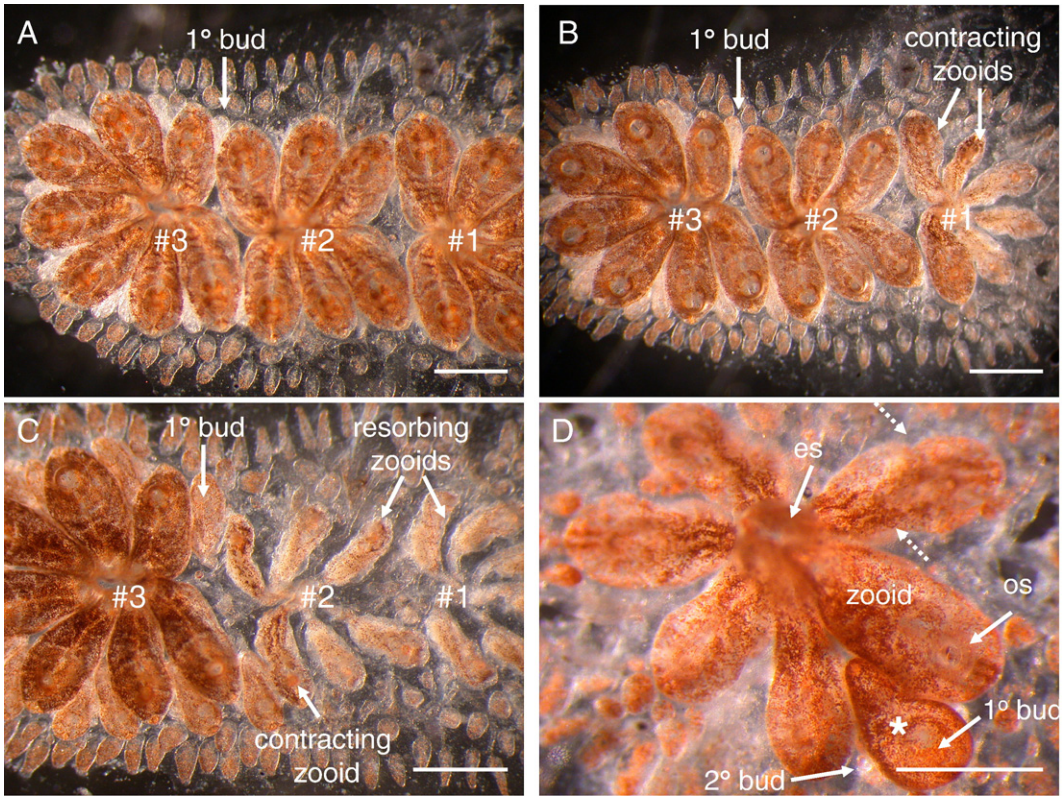

Fig. 7. Rescue from PCD onset in hemi-budectomized zooids is position-dependent. A Monterey *B. schlosseri* colony made up of three systems of zooids (N=7, 6, 6), in which buds were removed from the two systems on the right side (labeled #1 and #2, respectively) at stage B-1 of cyclic blastogenesis. Following surgery, 10 primary buds remained attached to the control system (labeled #3). All three systems were connected by extracorporeal blood vessels. (A) Appearance of the colony 48 h following hemi-budectomy, at stage C-1 of cyclic blastogenesis. Note that all zooids are inflated and actively filer-feeding. (B) Appearance of the colony 24 h later, at stage C2 of cyclic blastogenesis. The budectomized zooids from system #1 are contracting and have undergone siphon closure. (C) Appearance of the same colony, approximately 10 h later, in late C2 stage of cyclic blastogenesis. The budectomized zooids from system #2 have contracted, while zooids from system #1 are being resorbed. The control system of zooids (#3) initiated takeover approximately 11 h later. (D) Hemi-budectomized, single-system colony in which all buds were removed except one dextral (right) bud (asterisk). Note that only the zooid with its connected bud is inflated and filter feeding. All other zooids have either contracted (dashed arrows) and sunken into the colonial matrix following closure of their oral siphon. Panels A through D represent dorsal views. Abbreviations: 1° bud, primary bud; es, excurrent siphon; os, oral siphon. Scale bar indicates 1 mm.

tenet that, in *Botryllus*, zooid PCD is an intrinsic process that is suppressed by a survival signal whose expression requires mature buds. From a morphological standpoint, budectomized zooids underwent similar degenerative changes observed in control zooids, namely body wall contraction, siphon closure, loss of pigmentation and responsiveness to mechanical stimuli. Apoptotic cell corpses were also engulfed by blood phagocytes invading the peribranchial cavity, albeit with delayed kinetics, and resorption was correspondingly curtailed. These observations indicate that, even though buds are not functionally involved in activating PCD within adult zooids, their presence is required to carry out optimal zooid resorption during takeover and are entirely consistent with our earlier findings (Lauzon et al., 2002). Both Sabbadin (1956, 1958) and Berrill (1950) have argued that competition between asexual generations brings about the death of adult zooids during *Botryllus* takeover. Watkins (1958) investigated this question and demonstrated that, when all of the buds were removed in a colony, zooid lifespan was not significantly altered beyond the time of degeneration of control colonies. While these observations may seem somewhat discordant with our findings, it should be pointed out however that five colonies were budectomized in her study and these were carried out during the late stages of asexual development (Watkins, 1958; Berrill, 1961). Our findings unequivocally demonstrate that zooids budectomized in stage C-1 are refractory to early onset PCD. Consequently, the likeliest explanation is that Watkins must have carried out most of these budectomies in stage C-1 of cyclic blastogenesis or beyond. It should also be noted that, once vascular connections are severed between zooid systems in a multi-system colony, a gradual loss of synchronization in asexual development occurs between clonal replicate systems in subsequent blastogenic generations (Watanabe, 1953). The possibility remains that early onset zooid PCD following budectomy may be a consequence of desynchronization in cyclic blastogenesis. In addition, mechanical stress brought about from non-specific damage during the budectomy procedure could further curtail zooid lifespan. Several lines of evidence argue against either possibility as likely explanations for the early onset of zooid PCD in these colonies. First, unmanipulated control colonies whose vascular connections had been severed between zooid systems always retained blastogenic synchrony within the cycle in which observations were carried out. Second, all of the sham-operated colonies in

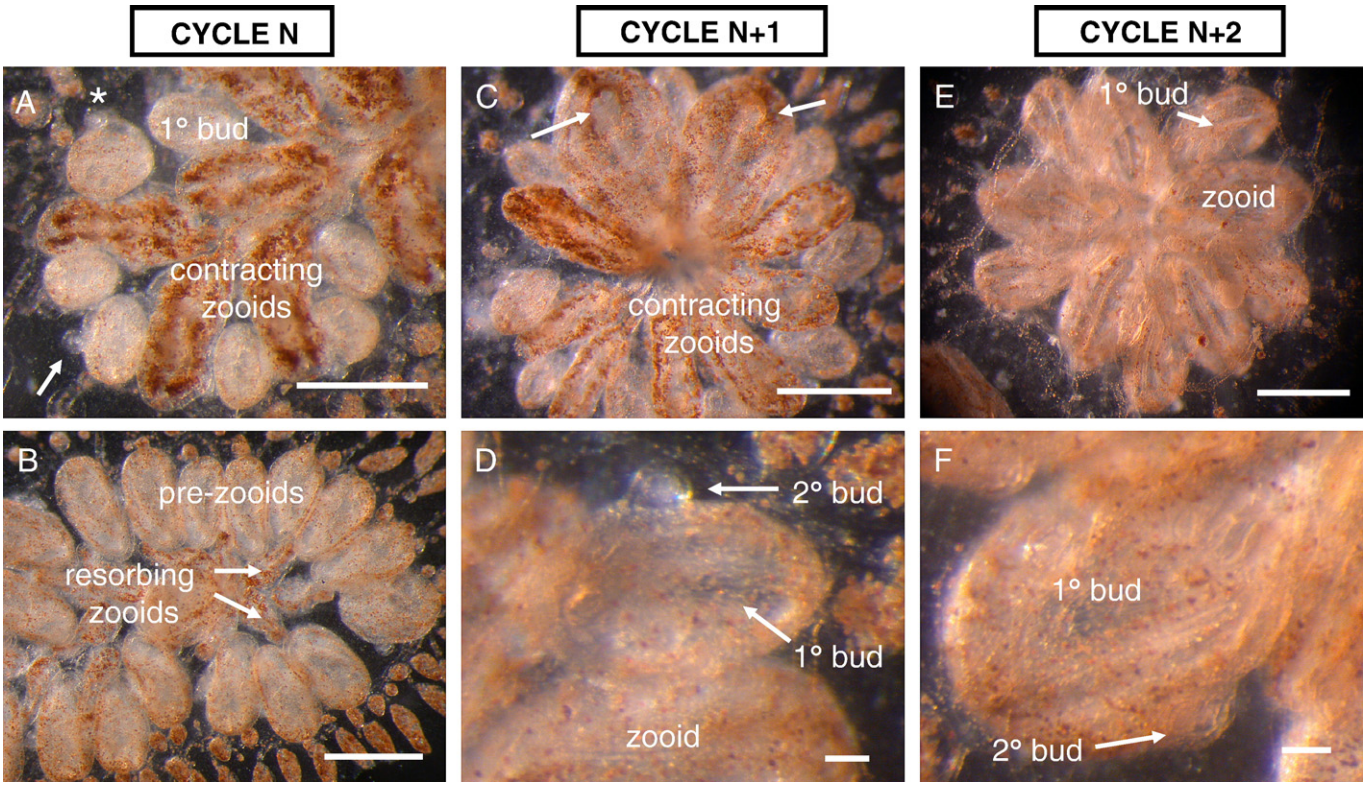

Fig. 8. Bud size regulates zooid lifespan in *B. schlosseri*. Monterey *B. schlosseri* colony (clone 1225c.11b) observed under stereomicroscopy over three consecutive blastogenic cycles: *N* (A and B), *N*+1 (C and D) and *N*+2 (E and F). This colony was approximately 22 months old at the time of observation. (A) Early onset of takeover during cycle *N*. The adult zooids have shutdown their oral and excurrent siphons and are contracting along their mantle wall. The secondary buds are underdeveloped, some being at the B-2 stage (asterisk) and most are in the early stages of organogenesis (arrow). (B) Appearance of the colony during stage D-3 of cycle *N*. The adult zooids are being resorbed normally, while the primary buds have become prefunctional zooids. (C) Asynchronous onset of takeover during cycle *N*+1. Seven zooids are undergoing contractions along their mantle wall, whereas two zooids are inflated and still filter-feeding (arrows indicate open oral siphons). Primary buds are also undersized. (D) Higher magnification of buds during asynchronous takeover of cycle *N*+1. The secondary bud in this example is underdeveloped, exhibiting the morphology of a double-layered, closed vesicle (stage B-2). (E) Appearance of the colony during late C-2 stage of blastogenic cycle *N*+2. The zooids are still fully inflated, and primary buds appear morphologically normal. (F) Higher magnification of buds during stage D-1 of blastogenic cycle *N*+2. The secondary bud has initiated organogenesis and exhibits well-developed subdivisions. Panels A–D and F represent dorsal views, whereas panel E is a ventral view. Abbreviations: 1° bud, primary bud; 2° bud, secondary bud. Scale bar indicates 1 mm (A, B, C and E) and 100 μm (D, F).

which tunic matrix, colonial vessels (marginal and connecting) and peduncular blood vessels between primary bud and zooid were severed underwent PCD synchronously with unmanipulated clonal replicates. Third, if inter-system desynchronization plays an important role in altering the timing mechanism of takeover, one would expect an equal number of budectomized colonies exhibiting an early or late onset of zooid PCD when compared to clonal replicate controls. Only early onset zooid PCD was observed in both Monterey and Woods Hole colonies subjected to complete budectomy. Fourth, budectomized zooids initiated mantle wall contraction and PCD during the organogenesis phase of secondary bud development (stage C-2), irrespective of the developmental stage at which buds had been removed from A-2 through B-2, as much as 4 days following budectomy. Collectively, these findings support the tenet that PCD suppression is a bud-dependent process.

A model of PCD regulation during cyclic blastogenesis

In multicellular animals, cell survival is often regulated by two antagonistic mechanisms: activation of the intracellular death

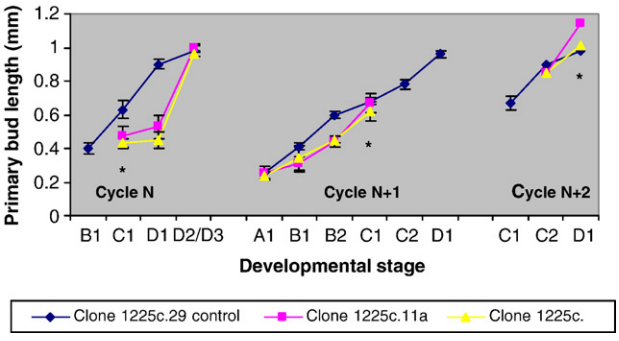

Fig. 9. Bud development during cyclic blastogenesis in *B. schlosseri*. Diameter measurements of primary buds for clonal replicates of colony 1225c (Monterey *B. schlosseri*) were carried out over three consecutive blastogenic cycles (*N*, *N*+1, *N*+2). Onset of takeover for clones 1225c.11a and 1225c.11b is indicated with an asterisk for each of the cycles. Clone 1225c.29 initiated takeover normally at stage D-1 for each of the three blastogenic cycles and thus served as an internal control. Clones 1225c.11a and 11b initiated early onset takeover in cycles *N* and *N*+1, but resumed normal takeover by cycle *N*+2. Each data point involved size measurements from at least 10 buds. Error bars represent standard deviation of the mean.

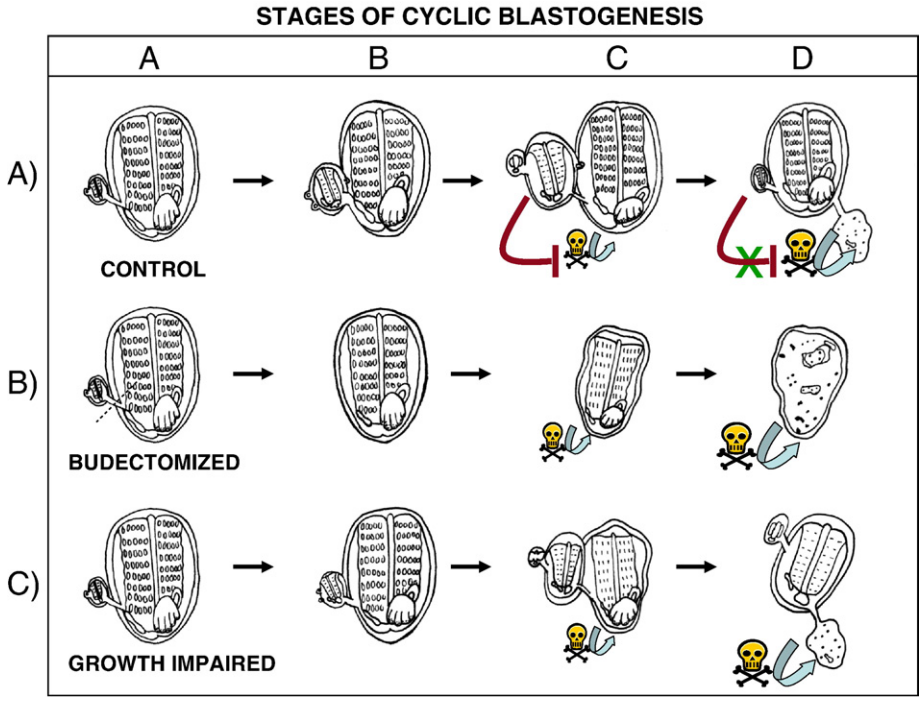

Fig. 10. Model of bud-mediated PCD suppression during cyclic blastogenesis in *B. schlosseri*. (A) Normal progression of the three overlapping asexual generations of individuals (adult zooid, primary and secondary bud) during stages A through D of cyclic blastogenesis. During the early phases of secondary bud organogenesis (stage C), a survival signal is sent by the primary bud which suppresses PCD activation in the adult zooid. Over the next 24 h, during the C to D stage transition, intensity of the death signal increases within adult zooids and eventually overrides the survival signal. Zooids are subsequently resorbed within 24–36 h. It is unknown how long the survival signal is expressed, and whether buds are also protected via PCD suppression or are simply refractory to the systemic death signal. (B) Following complete budectomy during stage A-2 (position of the cut is indicated by a dashed line), the zooid remains functional throughout stage B. However, in the absence of a survival signal originating from the primary bud, the zooid undergoes early onset PCD in early stage C-2, characterized by contraction of the mantle wall and closure of siphons (not illustrated). Macroscopic zooid resorption is also curtailed in budectomized zooids. (C) In old or physiologically impaired colonies, bud growth and morphogenesis are compromised thus affecting synthesis of the survival signal. Consequently, PCD suppression in adult zooids is curtailed, and onset of PCD occurs in early stage C. In these colonies, early PCD ultimately enables the colony to restore normal cyclic blastogenesis through recycling of zooid-derived cell corpses as raw materials for asexual budding and organismic regeneration.

program by extrinsic or intrinsic signals and cell death suppression by survival signals, as greater control may be achieved through the use of two signals instead of one (Raff, 1992; Coucouvanis and Martin, 1995; Jacobson et al., 1997; Coucouvanis and Martin, 1999). This balance gets offset when one type of signal overrides the other. There is precedent to believe that cell death regulation during asexual development in *Botryllus* may also be mediated in this fashion. Our findings are consistent with a model in which a zooid intrinsic (bud-independent) signal activates PCD systemically in zooid tissues and organs and is correspondingly antagonized by a bud-dependent survival signal which suppresses early onset zooid PCD (Fig. 10A). The competence of a zooid to undergo PCD is likely acquired during early secondary bud organogenesis as zooids budectomized between stage A-2 and B-2 always initiated PCD in stage C-2 of the blastogenic cycle. The PCD suppression mechanism also appears to be expressed in early stage C as zooids budectomized between stage C-1 and early C-2 were refractory to early onset PCD. Collectively, these observations suggest the following scenario: PCD suppression may only be feasible when levels of the cell death signal are low in early stage C-2 (Fig. 10A). As the concentration of the cell death signal increases between stage C and D, it may then override PCD suppression mediated by the survival signal to bring about normal onset of takeover. Bud-dependent PCD suppression may represent an essential homeostatic feature integrated into the physiology of colonial life in botryllid ascidians. Unlike the situation in vertebrates where only cells and tissues are replaced when needed, bodies in a *B. schlosseri* colony are transient feeding structures that function as niches supporting stem cell-dependent asexual budding and whose lifespan encompass three blastogenic cycles (Weissman, 2000; Lauzon et al., 2002). Thus, while each colony attempts to periodically produce functional zooids, a critical basis for survival in this animal is its ability to restart the budding cycle every week. We suggest that PCD suppression is central to the budding process: by maintaining the zooid's filter-feeding state as long as possible, the colony may be provided with additional energy resources that enable it to initiate a new round of stem cell-dependent organogenesis. In addition, this model provides a tangible explanation to our findings: in the absence of buds, cell death suppression is curtailed, resulting in early onset PCD in stage C-2 (Fig. 10B). Clearly, the continued presence of buds is not required for PCD suppression beyond stage C-1. Thus, another possibility may be that the survival factor is transiently expressed in stage C of cyclic blastogenesis. This alternative model would not necessitate an increase in the local concentration of the cell death activating signal. Our findings

do not enable us to distinguish between these possibilities at present. Moreover, since zooids selectively die during takeover, it is unclear if the developing buds are also protected via PCD suppression or whether they are simply refractory to cell death activation. These unanswered questions must await identification of the signaling molecule(s) involved in cell death regulation in *Botryllus*.

PCD suppression involves the colonial vasculature

The tissue or organ system involved in PCD suppression is unknown. However, our findings are consistent with the viewpoint that the survival signal acts via the colonial vasculature. In the great majority of two- and three-system colonies hemi-budectomized between stage A-2 and B-2 (49 of 70 colonies or 70%), PCD occurred simultaneously in both the budectomized and control zooid systems. Interestingly, budectomized zooids from the remaining 21 hemi-budectomized colonies underwent PCD in either mid (15.7%) or late (14.3%) stage C-2, but never in early C-2 as observed following complete budectomy. These results indicate that vascular connections between budectomized and control zooid systems have the ability to either completely or partially rescue early onset PCD. Partial rescue of early onset PCD was particularly intriguing in three-system hemi-budectomized colonies. PCD was consistently observed to first occur in the budectomized zooid system that was farthest away from the control zooid system in the colony. Furthermore, zooid PCD in single-system hemi-budectomized colonies in which a single bud remained occurred almost exclusively in a gradient fashion. The zooid that remained functional the longest was always the one joined directly to a primary bud through a vascularized epidermal peduncle. This connection presumably enabled it to be exposed to the highest concentrations of survival signal and could thus explain why it remained functional longer than other zooids. In contrast, PCD suppression was more effective in the hemi-budectomized colonies where bud dosage was higher. We have carried out partial budectomies using additional, single-system colonies by removing buds between every other zooid in each system. Onset of PCD in these colonies occurred synchronously in all zooids in concert with unmanipulated or sham-operated control clonal replicate controls (RJL, unpublished observations). The failure to observe a gradient of zooid PCD under these conditions indicates that the buds remaining following partial budectomy can fully compensate for the missing buds. Collectively, these observations strongly suggest that rescue from early onset PCD involves a short-range vascular signal whose activities regulate cell survival in a manner that is dependent on a zooid's proximity to a developing bud, as well as the number of buds present in a colony.

Blastogenic cycle duration is determined by the physiological state of the colony

If PCD suppression represents a critical feature of colonial life in *Botryllus*, one may ask: what regulates the regulator? Is there a sensing mechanism that controls temporal expression patterns of cell survival and PCD activation signals? If so, what might it be? It is possible that intra-colony energy stores may serve as cues that regulate the duration of each blastogenic cycle. If a colony is growth-impaired either from advancing blastogenic age (Rinkevich et al., 1992; Lauzon et al., 2000) or inadequate food supply in its environment (Sabbadin, 1956; Sabbadin, 1958), bud growth and morphogenesis may be correspondingly impeded. This, in turn, could prevent the bud from reaching a developmental state that would enable it to exert its PCD suppressive function, thus triggering early onset of zooid PCD (Fig. 10C). Our findings are consistent with this viewpoint. Over the course of several years, we have repeatedly observed altered blastogenic cycles in older laboratory-raised colonies (RJL, unpublished observations). The colony described in this study (1225c) displayed undersized and under-developed buds and simultaneously initiated early onset takeover for the first two blastogenic cycles during which observations were conducted. By the third blastogenic cycle, a normal bud growth pattern and cycle duration had been restored. These intriguing findings lead us to ask what benefit early onset zooid PCD may provide a colony. Here, the parallels between iatrogenic budectomy and growth impairment induced by age or nutrient deprivation may offer clues. The common phenotype observed under both conditions (i.e. early onset zooid PCD) may be representative of the same underlying PCD regulatory mechanism that is expressed in a stage-specific fashion during cyclic blastogenesis in *Botryllus*. The presence of small, under-differentiated buds would be expected to confer a similar phenotype as absence of buds resulting from complete budectomy. Both situations would effectively curtail expression of the survival signal below the required threshold for cell death suppression, resulting in early onset zooid PCD. We have previously demonstrated that the single buds remaining following hemi-budectomy in single-system colonies grew to significantly larger size at functional maturity and exhibited hyperplasia (Lauzon et al., 2002). Conversely, if colonies were zooidectomized at the onset of takeover following siphon closure, bud growth and zooid number in a colony were significantly reduced. Since no adult individuals remained following zooidectomy, we concluded that intra-colony resources were likely providing the energy used in organismic regeneration. These collective observations led us to propose the existence of a colony-wide recycling mechanism during takeover, in which components of the zooid viscera are engulfed by blood phagocytes and reutilized to build new organisms on a weekly basis (Lauzon et al., 2002). Thus, it is conceivable that, when energy stores are low because of the physiological age of the colony (this study) or through nutritional deprivation (Sabbadin, 1956, 1958), asexual budding could be boosted from early onset zooid PCD and recycling. Conversely, when intra-colony energy stores are adequate, bud development occurs normally and the zooids are maintained in a functional state longer via PCD suppression. Collectively, our findings are consistent with Sabbadin's (1994) proposal that *B. schlosseri* colonies are highly integrated, homeostatic systems that can accommodate a wide range of intrinsic and environmental conditions by altering zooid

number as well as number of coexisting generations with each blastogenic cycle. The dependence on a survival signal during cyclic blastogenesis in botryllid ascidians may thus underscore a life history strategy that is ultimately geared at optimizing colony lifespan and fecundity in the field.

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